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Resistance to Linezolid Caused by Modifications at Its Binding Site on the Ribosome

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Linezolid is an oxazolidinone antibiotic in clinical use for the treatment of serious infections of resistant Gram-positive bacteria. It inhibits protein synthesis by binding to the peptidyl transferase center on the ribosome. Almost all known resistance mechanisms involve small alterations to the linezolid binding site, so this review will therefore focus on the various changes that can adversely affect drug binding and confer resistance. High-resolution structures of linezolid bound to the 50S ribosomal subunit show that it binds in a deep cleft that is surrounded by 23S rRNA nucleotides. Mutation of 23S rRNA has for some time been established as a linezolid resistance mechanism. Although ribosomal proteins L3 and L4 are located further away from the bound drug, mutations in specific regions of these proteins are increasingly being associated with linezolid resistance. However, very little evidence has been presented to confirm this. Furthermore, recent findings on the Cfr methyltransferase underscore the modification of 23S rRNA as a highly effective and transferable form of linezolid resistance. On a positive note, detailed knowledge of the linezolid binding site has facilitated the design of a new generation of oxazolidinones that show improved properties against the known resistance mechanisms.

LINEZOLID

Linezolid (Fig. 1A) is a synthetic drug and a member of the oxazolidinone class of antibiotics. It acts as a protein synthesis inhibitor by binding to the ribosomal peptidyl transferase center (PTC) and stopping the growth of bacteria. Linezolid appeared on the market in 2000 for treatment of serious infections caused by Gram-positive bacteria resistant to other antibiotics, including streptococci, vancomycin-resistant enterococci (VRE), and methicillin-resistant *Staphylococcus aureus* (MRSA) (9). It is not well suited for fighting Gram-negative pathogenic bacteria that are intrinsically resistant due to efflux pumps that force linezolid out of the cell faster than it can accumulate (1, 74). Linezolid is one of the few truly new antibiotics that have been introduced in many years, as most newcomers are derivatives of existing drugs. The development of new antibiotics is apparently not an attractive business despite the expanding problem of antibiotic resistance in pathogenic bacteria. At the introduction of linezolid, it was claimed that there would be no cross-resistance to linezolid and that resistance would be rare and difficult for the bacteria to develop (20, 103), but resistance does appear (reviewed in references 61 and 85). This minireview will present in detail how several types of modifications at the linezolid binding site on the ribosome can cause resistance to linezolid and other oxazolidinones. The only clear nonribosomal linezolid resistance mechanism reported is related to mutations causing increased expression of ABC transporter genes in *Streptococcus pneumoniae* (3, 19). It has also been shown that *Staphylococcus aureus* possesses a gene for a major-facilitator-superfamily-type multidrug efflux pump named LmrS that is capable of extruding linezolid (21).

Although linezolid is currently the only oxazolidinone antibiotic approved for treatment of bacterial infections, other oxazolidinones have been investigated and have entered development. Some examples are eperezolid, which behaves similarly to linezolid (reviewed in reference 9) and posizolid (AZD2563) (98). Some newer derivatives are tedizolid phosphate (previously known as torezolid phosphate [TR-701]), an oxazolidinone drug currently in phase III trials for acute bacterial skin and skin structure infections, including those caused by MRSA (http://www.clinicaltrials.gov/ct2/show/NCT01170221?term=tedizolid&rank=4) (79), and radezolid (RX-1741), which has completed two phase II clinical trials, one for uncomplicated skin and skin-structure infections and the other for community-acquired pneumonia (86). There is an obvious interest in developing a new generation of oxazolidinone derivatives which can overcome the known resistance mechanisms. A recent paper (52) focused on the structure-activity relationships (SAR) of oxazolidinones, including some new derivatives, using a panel of clinical and laboratory-derived *S. aureus* strains possessing ribosomal resistance mutations or the Cfr methyltransferase gene. The data show that improvement regarding the known resistance mechanisms is possible for oxazolidinone derivatives.

THE LINEZOLID BINDING SITE ON THE RIBOSOME

Although some of the first reports on linezolid claimed that it was a unique drug with a unique mechanism of action, we now know that the mechanism of action is similar to those of other antibiotics binding to the PTC. The first indications of the exact binding site of linezolid on the ribosome came in 1999 from studies of resistance mutations in 23S rRNA and pointed to the PTC (41). The PTC is in the middle of the 50S ribosomal subunit in the bottom of the cleft where the 3′ ends of aminoacyl-tRNA and peptidyl-tRNA are positioned for peptide transfer (Fig. 1B and C). Binding of linezolid to this area was subsequently supported by other mutagenesis studies but could not be verified by antibiotic

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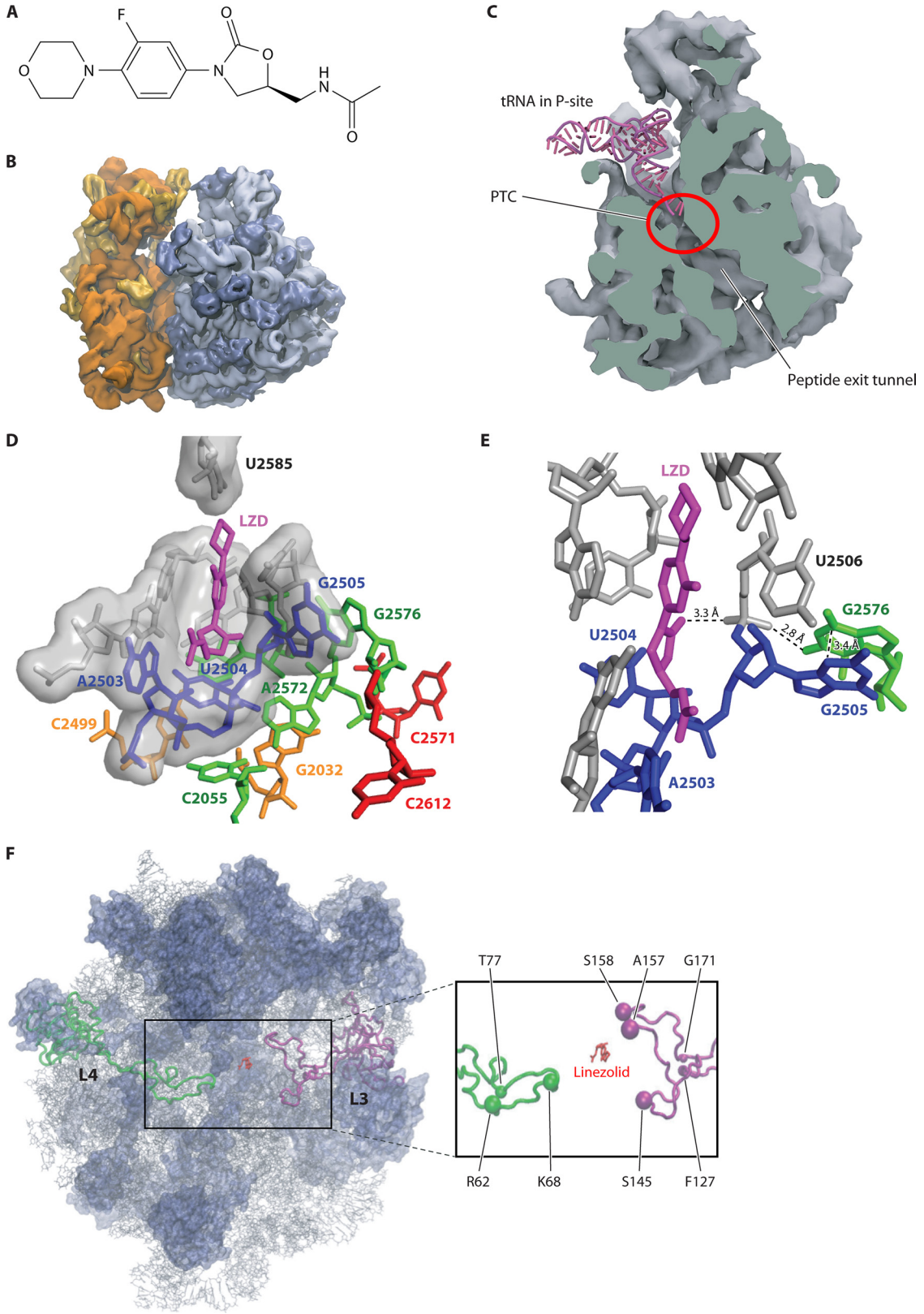

FIG 1 (A) The chemical structure of linezolid. (B) The *E. coli* 70S X-ray structure with 16S rRNA in orange, 30S ribosomal proteins in yellow, 23S rRNA in gray, and 50S ribosomal proteins in blue (PDB file from reference 75). (C) A cutaway view of the large ribosomal subunit with a red circle at the PTC (PDB file from reference 77). A P-site-bound tRNA is shown in magenta, and the PTC and peptide exit tunnel are indicated. (D and E) Close-up views of the linezolid binding site made with the PyMOL software program. The coloring of nucleotides indicates first-layer (blue), second-layer (green), third-layer (orange), and outer-layer (red) nucleotides with respect to linezolid. (D) The locations of mutated nucleotides with respect to bound linezolid. First-layer nucleotides are shown in surface representation. (E) The position of G2576 with respect to linezolid. (F) Illustration of how parts of the L3 (in purple) and L4 (in green) ribosomal proteins extend toward the PTC where linezolid (in red) is bound. Selected amino acids are marked with the corresponding *S. aureus* numbering. The coordinates are from PDB file 3DLL (95).

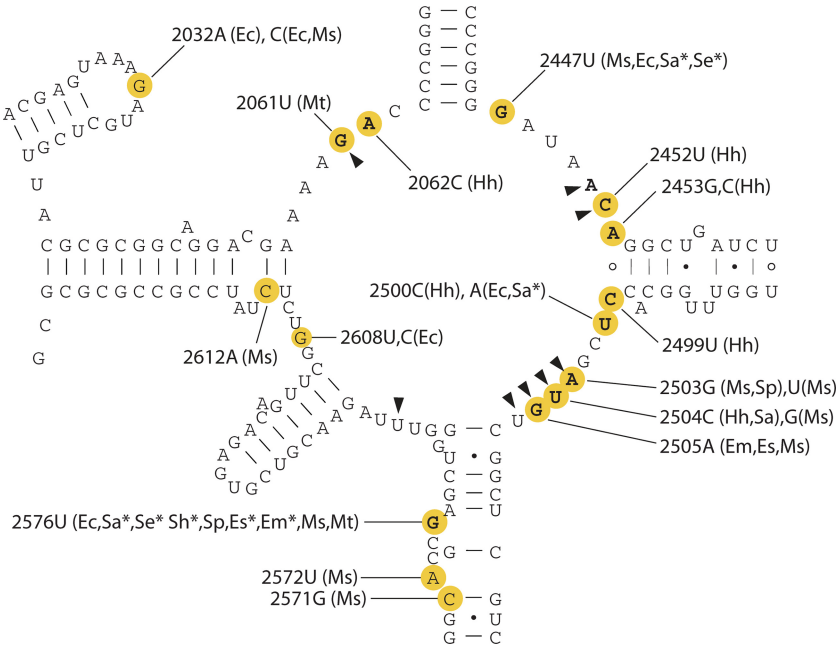

FIG 2 Secondary structure of the peptidyl transferase loop of domain V of 23S rRNA (*M. smegmatis* sequence in *E. coli* numbering). The nucleotides that form the linezolid binding pocket are indicated with black triangles. Nucleotide positions where mutations confer linezolid resistance are marked with yellow circles. The nucleotides where mutations have a significant effect on linezolid MIC (>4-fold MIC increase) are in bold type, whereas those where mutations have a small to moderate effect on the linezolid MIC (4-fold or less MIC increase) are in regular type. The mutations and corresponding organisms are indicated with two-letter abbreviations: Ec (*E. coli*), Sa (*S. aureus*), Se (*S. epidermidis*), Sh (*S. haemolyticus*), Sp (*S. pneumoniae*), Es (*E. faecalis*), Em (*E. faecium*), Ms (*M. smegmatis*), Mt (*M. tuberculosis*), and Hh (*H. halobium*). Asterisks indicate mutations found in clinical isolates. Only mutations where some evidence or strong indication of the mutation-resistance relationship has been published are marked on the figure (8, 19, 30, 41, 47, 50, 56, 59, 60, 62, 66, 69, 73, 97, 99, 100).

footprinting, a technique that has been used successfully to localize ribosomal binding sites for other antibiotics. Cross-linking studies with linezolid derivatives also pointed to a location at the PTC (12, 46). In 2008, this site was confirmed by crystal structures of linezolid bound to the 50S ribosomal subunit from the archaeon *Haloarcula marismortui* (36) and from the bacterium *Deinococcus radiodurans* (95). The structures reveal that linezolid binds to the A site of the PTC and interacts extensively with many 23S rRNA nucleotides in the neighborhood (Fig. 1D and E and Fig. 2). The PTC is almost exclusively composed of RNA, and the nucleotides in this region are phylogenetically highly conserved. The many crystal structures of 50S subunit-antibiotic complexes that have now been published also reveal that linezolid partially shares its binding site with other antibiotics that inhibit protein synthesis. Chloramphenicol, clindamycin, tiamulin, and streptogramin A (plus bruceantin, homoharringtonine, and anisomycin, which preferentially bind to eukaryotic and archaeal ribosomes) all occupy overlapping sites with linezolid at the A site of the PTC (original references and Protein Data Bank [PDB] identification [ID] are in references 10, 14, and 94), where the aminoacyl moiety of A-site tRNA has to be positioned for successful peptide transfer. This is underscored by the cross-resistance between linezolid and other drugs binding to this region. Thus, linezolid binds to a common antibiotic site on the ribosome and acts by blocking precise positioning of A-site tRNA in the PTC. The crystal structures have also shown that the nucleotides in the PTC adapt to the binding of antibiotics by slight changes in their relative positions, where especially nucleotide 2585 (Fig. 1D) seems very disposed to repositioning (95). The differences in modes of action of the antibiotics must be ascribed to their overlapping but not identical binding sites and how they affect neighboring nucleotides in combination with their mode of access, affinity for the site, and association and dissociation rate constants.

Knowledge of the molecular details of linezolid binding to the PTC is highly advantageous for development of new oxazolidinone derivatives. It can indicate where there is room for drug derivatization, suggest sites for additional interactions, and thus facilitate the prediction of beneficial as well as detrimental interactions. Finally, the structures can be used to suggest drug modifications that will allow it to bind to the ribosome despite the presence of resistance determinants (22). This approach has been successfully exploited by the company Rib-X, who used knowledge about linezolid and sparsomycin binding to the 50S ribosomal subunit to create new drugs from parts of these antibiotics (86). They bridged and derivatized the two components, and one example is the radezolid mentioned above, where one ring of the linezolid (left side on Fig. 1A) is replaced and extended to obtain additional interaction in the PTC. Another example is presented by Trius Therapeutics, who modeled linezolid and tedizolid (previously known as torezolid [TR-700]) to the binding sites in the PTC with and without a methylation at the C-8 position of the adenine at position 2503 (52). This showed a better accommodation of TR-700 than linezolid in the presence of the methylation, which is the resistance determinant mediated by the Cfr methyltransferase (described below).

RESISTANCE CAUSED BY 23S rRNA MUTATIONS

The linezolid binding site at the PTC is composed entirely of RNA, and until recently, mutation of 23S rRNA was the only known linezolid resistance mechanism. The binding pocket is lined with

TABLE 1 23S rRNA linezolid resistance mutations and corresponding nucleotide-linezolid distances

23S rRNA mutation[a]	*D. radiodurans* position[b]	LZD distance (Å)[c]	Origin[d]	Organism(s)[e]	Reference(s)
G2032A	G2015	9.3		Ec	99
G2032C	G2015	9.3		Ec, Ms	56, 99
G2061U	G2044	3.0	S	Mt	30
A2062C	A2045	8.1	S	Hh	41
G2447U	G2426	6.2	C, S	Ec, Ms, Sa, Se	50, 66, 73, 97, 99
C2452U	C2431	2.9	S	Hh	41
A2453G	A2432	6.1	S	Hh	41
A2453C	A2432	6.1	S	Hh	41
C2499U	C2478	8.1	S	Hh	41
U2500A	U2479	7.2	C, S	Ec, Sa	62, 66
U2500C	U2479	7.2	S	Hh	41
A2503G	A2482	2.2	S	Ms, Sp	19, 56
A2503U	A2482	2.2		Ms	47
U2504C	U2483	4.5	S	Hh, Sa	41, 50
U2504G	U2483	4.5		Ms	56
G2505A	G2484	2.4	S	Em, Es, Ms	8, 56, 69
C2571G	C2550	14.7		Ms	56
A2572U	A2551	6.6		Ms	56
G2576U	G2555	7.9	C, S	Ec, Em, Es, Ms, Mt, Sa, Se, Sh, Sp	19, 30, 50, 56, 59, 60, 66, 69, 97
G2608U	G2588	15.9		Ec	100
G2608C	G2588	15.9		Ec	100
C2612A	C2591	17.7		Ms	56

[a] The nucleotide positions of the mutations are listed according to *E. coli* numbering.
[b] The corresponding nucleotide positions in *D. radiodurans* 23S rRNA.
[c] The closest approximate distances are given between the corresponding *D. radiodurans* nucleotides and linezolid (LZD) from the *D. radiodurans* 50S-linezolid complex X-ray structure (PDB ID 3DLL) (95).
[d] Indicates whether a mutation was obtained via *in vitro* selection (S) or found in clinical isolates (C).
[e] Abbreviations: Ec, *E. coli*; Sa, *S. aureus*; Se, *S. epidermidis*; Sh, *S. haemolyticus*; Sp, *S. pneumoniae*; Es, *E. faecalis*; Em, *E. faecium*; Ms, *M. smegmatis*; Mt, *M. tuberculosis*; Hh, *H. halobium*.

the universally conserved nucleotides G2061, A2451, C2452, A2503, U2504, G2505, U2506, and U2585, which interact directly with linezolid (95) (Fig. 1D and 2). Strains selected for linezolid resistance have mutations in 23S rRNA nucleotides G2061, C2452, A2503, U2504, and G2505, which abut the bound linezolid molecule (Fig. 1D and E and Table 1), but also at nucleotides that are located more distally, such as A2062, G2447, A2453, C2499, U2500, and G2576 (11, 19, 30, 41, 50, 51, 66, 69, 73) (Fig. 1D and E and Table 1). The pattern of resistance is organism specific in that the obtained mutation sites differ, with only little overlap, between *Halobacterium halobium*, *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *S. aureus*, and *S. pneumonia*e (Fig. 2 shows detailed information). This specificity is likely related to single-base identity differences in the RNA nucleotides that are more distant from and do not directly abut the bound linezolid molecule and variations in the fitness costs associated with specific mutations in different organisms. The degree of resistance conferred by the mutations is not a simple function of the nucleotide-linezolid distance, and mutations at distal nucleotides that do not interact with linezolid directly, in particular, G2576U (19, 30, 56, 66, 69, 97) (Fig. 1D and E) and G2447U (66, 73, 97, 99), confer significant resistance (Table 1).

Although the effect of a few engineered 23S rRNA mutations on linezolid susceptibility has been investigated in *E. coli* by expressing the mutated rRNA on a multicopy plasmid (99), the multiplicity of rRNA operons and heterogeneous ribosome populations complicates the study of rRNA mutations in many bacteria. Additional mutations have been investigated in *M. smegmatis* strains modified to contain a single rRNA operon (31, 32, 47, 57), where one model system allows for the introduction of mutations regardless of whether the mutations confer antibiotic resistance or not (32). This genetic system has been used to test whether rRNA mutations that confer linezolid resistance in other organisms (G2576U) also do so in *M. smegmatis* and to validate rRNA mutations associated with linezolid resistance in the literature without genetic proof (A2503G, G2505A, C2571G, and C2612A) (56). The study revealed that mutations at some distance from an antibiotic binding site can confer resistance and that the same rRNA mutation can have significantly different effects in different bacteria. Moreover, the system has been used to introduce the double rRNA mutations G2032A-C2499A, G2032A-U2504G, C2055A-U2504G, and C2055A-A2572U and show that there are strong synergistic effects on linezolid resistance relative to the effects of the corresponding single rRNA mutations (56).

The most frequently reported mutation in linezolid-resistant clinical isolates is 23S RNA G2576U. This mutation has been reported in both staphylococci and enterococci (6, 15, 34, 40, 59, 61, 70, 71, 87, 97, 101), and a clear correlation between the number of mutated rRNA operons and the linezolid MIC has been found (2, 35, 59). Most reports of the G2576U mutation in clinical isolates are associated with some form of increased or prolonged linezolid treatment or with high linezolid usage at the local hospital (29, 84), underscoring the importance of judicious use of linezolid in clinical settings. Although some studies have documented reversion of the G2576U mutation in the absence of linezolid selection

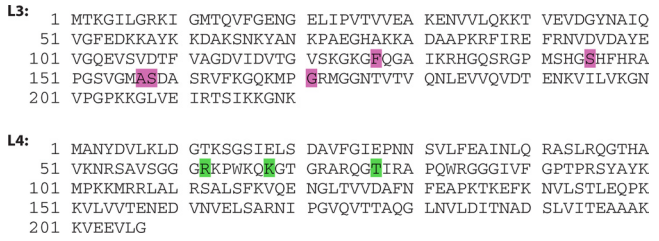

FIG 3 The sequences of 50S ribosomal proteins L3 and L4 from *Staphylococcus aureus* MRSA252. The amino acids highlighted in purple and green correspond to those shown as balls in Fig. 1F. For the L3 sequence, A157 is the equivalent of *E. coli* L3 N149.

(62, 87), a recent report showed that the G2576U mutation was retained in a *Staphylococcus haemolyticus* isolate even after 30 passages on antibiotic-free medium (60).

The U2500A (62) and G2447U (97) 23S RNA mutations have been reported in linezolid-resistant clinical isolates of staphylococci, and these mutations have been shown to confer linezolid resistance in *in vitro*-selected mutants of *E. coli* and/or *M. smegmatis* (66, 73). Although the U2504A mutation has been reported in clinical staphylococcal isolates (16, 48, 97), the only U2504 mutation isolated from *in vitro* selection with linezolid to date is U2504C (41, 50). Additional mutated positions of 23S rRNA at G2603U (49, 78, 83) and C2534U (97) have been reported in clinical isolates with reduced linezolid susceptibility, but a direct relationship between these mutations and linezolid resistance is unclear.

LINEZOLID RESISTANCE AND A TENTATIVE RELATIONSHIP TO MUTATIONS IN RIBOSOMAL PROTEINS L3 AND L4

Another type of linezolid resistance determinant has received attention recently, namely, mutations in the ribosomal L3 protein. The main part of ribosomal protein L3 is positioned on the surface of the 50S subunit, but a loop ending in two tips extends into the PTC (Fig. 1F and Fig. 3). Bacterial L3 mutations have, since 2003, been associated with resistance to linezolid, tiamulin/valnemulin, and anisomycin, which all bind to overlapping sites at the PTC. The first L3 resistance mutation in bacteria was detected by us in *E. coli* by selection with tiamulin, and its role in resistance was verified by genetic evidence (7). Many studies have since associated L3 mutations with linezolid resistance in *S. aureus*, *Staphylococcus cohnii*, and *Staphylococcus epidermidis*, and the data are summarized in Table 2.

Locke and colleagues (54) performed a selection study with both methicillin-sensitive and MRSA strains and the oxazolidinones linezolid and TR-700. They obtained various mutations in 23S RNA (see above) as well as mutations in L3 and L4 (Table 2), and apparently only the 23S RNA mutations give high resistance. As the authors note, directed mutagenesis and heterologous expression studies are needed to conclusively link these mutations to oxazolidinone resistance. Nevertheless, the positions of the mutations are correlated with the linezolid binding site in an X-ray structure and a possible mechanism of structural perturbations is explained, which, together with the absence of mutations in other remote peptide regions and in L22 (also sequenced), points to a role in resistance development.

A search for similar mutations by the same authors in clinical staphylococci revealed a linezolid-resistant *S. aureus* strain with an L3 mutation and a linezolid-resistant *S. epidermidis* strain with an L3 mutation plus the G2447U 23S rRNA mutation (53). Another study identified L3 mutations in the region near the PTC in linezolid-resistant *S. aureus* strains from a hospital outbreak in Spain that also harbored the *cfr* gene (55). Similarly, sequencing of selected genes from three clinical isolates from Mexico, with *cfr* and linezolid resistance, also revealed L3 and L4 mutations (63) (Table 2), but the effects of the mutations were not confirmed. Two of these strains harbored an L101V L3 mutation that has also been detected in a sensitive control strain and is therefore not considered relevant for linezolid resistance. Ten *S. epidermidis* strains from Italy with reduced linezolid susceptibility were investigated by sequencing the 23S rRNA gene and ribosomal protein L3, L4, and L22 genes, as well as looking for the *cfr* gene (65). It was concluded that the L3 mutations F147L and/or A157R appear to be responsible for the elevated linezolid MIC values, as adjacent alterations have been associated with resistance in other strains. Another recent study (43) reported multiple L3 mutations, but in most cases, the mutations were together with the 23S rRNA mutation G2576T, which is known to confer linezolid resistance, and with an L4 insertion. Finally, two new L3 mutations have also been found in linezolid-resistant *S. aureus* strains from a cystic fibrosis patient after prolonged drug treatment, where one strain also possessed the G2576U 23S rRNA mutation (15). It is worth mentioning that mutations of L3 at some of the positions mentioned above, as well as at amino acids close by, are associated with resistance to the pleuromutilins retapamulin and tiamulin (24, 42, 43, 66). As linezolid and the pleuromutilins binds at overlapping sites at the PTC, these findings support the relationship between L3 mutations and PTC antibiotic resistance in general.

Part of ribosomal protein L4 is also placed relatively close to the PTC (Fig. 1F) but in the tunnel through which nascent peptides exit the ribosome. A surveillance study found a slightly linezolid-resistant *S. pneumoniae* isolate with a six-nucleotide deletion in the L4 gene (ΔW65-R66) but with no genetic proof presented (18). Another surveillance study identified an *S. pneumoniae* strain with the same deletion plus a strain with a neighboring six-nucleotide deletion (ΔK68-G69) in the L4 gene. The deletions caused a slightly reduced susceptibility to linezolid (MIC change from 1 μg/ml to 4 μg/ml) as evidenced by transformations and were associated with a fitness cost (96). The amino acid deletions are in the same region as mutations known to be involved in macrolide resistance (26), and as macrolide antibiotics bind to a site neighboring but not directly overlapping the linezolid binding site, the effect of the deletions is probably caused by an allosteric mechanism.

Four different mutations were found in the L4 ribosomal protein in another study of linezolid-resistant *S. epidermidis* isolates (97), but two of these mutations occurred in isolates that also harbored 23S rRNA mutations. The N158S mutation has been found among linezolid-sensitive *S. epidermidis* isolates and is therefore probably a clonal marker rather than a resistance mutation (97). Also, it has been concluded that L4 K68R is not responsible for resistance in an *S. epidermidis* study, as it did not increase resistance when present together with other mutations (65). In contrast, the L4 mutation K68Q found in the *S. aureus* selection study mentioned above was assumed to play a role in resistance (54), as was an L4 gene mutation in *Clostridium perfringens* (33). The L4 mutations related to linezolid resistance are summarized in Table 2, but the data presented do not present a consistent

TABLE 2 Mutations in L3 and L4 that have been associated with linezolid resistance in staphylococci (and one case in *Clostridium perfringens*) and corresponding amino acid-linezolid distances[g]

Mutation	Organism	*D. radiodurans* L3[a]	LZD distance[b]	Remarks[c]	Reference
L3					
ΔF127-H146	*S. aureus*	T113-K132			54
G139R	*S. aureus*	G125	>25	C, T, 2576T	15
ΔS145	*S. aureus*	S131	>25	C	53
ΔS145/H146Y	*S. aureus*	S131/K132	>25/19.3	C, cfr	55
H146R/M156T	*Staphylococcus*	K132/G143	19.3/15.2	C, T, 2215A, 2576T, —,[d] L4-ins70G	43
H146Q/V154L/A157R	*Staphylococcus*	K132/I141/R144	19.3/22.1/7.0	C, T, —,[d] L4-ins70G, —[e]	43
F147I	*Staphylococcus*	K133 or W134	22.4 or >25	C, T, 2215A, 2576T, —,[d] L4-ins70G	43
F147L/A157R	*S. epidermidis*	K133 or W134/R144	22.4 or >25/7.0	C, —,[d] —,[e] L4-K68R/[e]	65
G152D	*S. aureus*	G139	20.4	2447T	54
G152D	*S. aureus*	G139	20.4	C, T	15
G155R	*S. aureus*	G142	17.5		54
G155R/M169L	*S. aureus*	G142/M156	17.5/>25		54
A157R	*S. epidermidis*	R144	7.0	C, 2447T	53
S158F/D159Y	*S. epidermidis*	K145/T146	13.3/5.4	C, cfr, L3-L101V	63
S158Y/D159Y	*S. cohnii*	K145/T146	13.3/5.4	C, cfr, L4-N20S/A133T/V155I	63
ΔM169-G174	*S. aureus*	DM156-G161		C, cfr	55
L4					
N20S/A133T/V155I	*S. cohnii*	—[f]		C, cfr, L3-S158F/D159Y	63
ΔW65-R66	*S. pneumoniae*	Y59-G60	24.2-21.6		18
ΔW65-R66	*S. pneumoniae*	Y59-G60	24.2-21.6	C, RE	96
ΔK68-G69	*S. pneumoniae*	K62-Q63	11.2-15.1	C, RE	96
K68N	*S. epidermidis*	K62	11.2	2576T	97
K68Q	*S. aureus*	K62	11.2		54
G71D	*Clostridium perfringens*	G65	15.6		33
ins71GGR72	*S. epidermidis*	G65/N66	15.6/16.1	2576T	97
L108S/ins71GGR72	*S. epidermidis*	L102	>75	2534T, —[e]	97

[a] The L3 sequence from the *D. radiodurans* 50S X-ray structure (95) used for distance determination has been aligned with L3 from *S. aureus*, and the amino acids corresponding to the mutations are listed.

[b] The distance is the smallest distance from linezolid (LZD) to the corresponding *D. radiodurans* amino acid.

[c] Selected additional information. C, from clinical isolates; T, treatment with linezolid; cfr, containing the *cfr* gene; RE, resistance evidence. Additional resistance determinants are shown (xxxxN refers to 23S RNA positions corresponding to *E. coli* 23S RNA, and L3- and L4- indicate additional mutations). See the original references for more details.

[d] —, L3-L101V, which is not expected to influence linezolid resistance.

[e] —, L4-N158S, which is not expected to influence linezolid resistance.

[f] No data for alignment were available.

[g] Mutations with indications that they are merely strain markers and not relevant for antibiotic resistance are not included, except where they are found together with other mutations and are then posted under Remarks. The resistance effects vary from very minor effects to substantial resistance, and only in a few cases has there been direct evidence linking the mutations to resistance. The slash is used when there is more than one mutation. "K133 or W134" indicates that the alignment did not reveal which of these two positions corresponds to F147.

pattern and it is not definitively established which changes contribute directly to linezolid resistance.

As sequencing of genes from bacteria exhibiting resistance becomes common, many mutations are revealed, but they might not all be related to the observed resistance. Some mutations could be just random changes without significant effect, while others are likely related to either causing the resistance or accommodating or sustaining resistance in a synergistic way. This is supported by the fact that several of the L3 and L4 mutations observed in relation to linezolid resistance are found together with mutations in 23S rRNA at or near the PTC as mentioned above. Similar possible synergistic effects have also been reported for other PTC antibiotics in other bacteria such as *M. smegmatis* (56) and *Brachyspira* spp. (28, 68), indicating an interplay between multiple mutations in relation to resistance, accommodation of mutations, and fitness cost. More specific information about the effects of the single and combined mutations is needed to clarify their detailed interactions.

RESISTANCE CAUSED BY ALTERATIONS IN 23S rRNA MODIFICATION

It is well established that RNA modifications placed at or near an antibiotic binding site can affect drug binding to the ribosome. rRNA is intrinsically modified with methyl groups and pseudouridine residues, mediated by methyltransferase and pseudouridine synthase enzymes, respectively. The modifications are clustered at functional centers on the ribosome and have a collective importance in optimizing different steps of protein synthesis, although the exact roles of each modification are not well described. Modifications can also be acquired, but to date, the only type of modification that provides acquired antibiotic resistance is methylation. Thus, resistance generally occurs either by the inactivation of an indigenous methyltransferase or by the acquisition of an antibiotic resistance methyltransferase.

Several housekeeping modifications at the peptidyl transferase center are known to affect linezolid susceptibility. The pseudouridylation of 23S rRNA nucleotide 2504 confers resistance to lin-

ezolid, clindamycin, and tiamulin, suggesting that this modification may have evolved as an intrinsic resistance mechanism to protect bacteria from PTC antibiotics (88). Inactivation of the spr0333 methyltransferase targeting G2445 in 23S rRNA results in decreased susceptibility to linezolid in *S. pneumoniae* (3, 19). Likewise, mutations inactivating the methyltransferase RlmN, which methylates the C-2 position of A2503 of 23S rRNA, also lower linezolid susceptibility in *S. aureus* (23, 44, 88).

The only known transferable form of linezolid resistance is conferred by the multiresistance gene *cfr*, which encodes an rRNA methyltransferase (39). Cfr adds a methyl group at the C-8 position of 23S rRNA nucleotide A2503 (25), the only example of this modification found in natural RNAs so far. The methylation confers combined resistance to five different classes of antibiotics that bind at overlapping nonidentical sites at the PTC (58). The resistance is substantial and functions in both Gram-positive and Gram-negative bacteria. The phenotype is called $PhLOPS_A$, for resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A antibiotics. In addition, Cfr confers significant resistance to selected 16-membered ring macrolide antibiotics such as josamycin and spiramycin, but not tylosin (81). The methylation is positioned in the PTC, and its direct interference with drug binding is supported by X-ray structures of linezolid bound to the *D. radiodurans* and *H. marismortui* 50S subunits (36, 95).

Cfr is related to the RlmN methyltransferases, which add a methyl group at the C-2 position of 23S rRNA nucleotide A2503 (90). Although the primary activity of Cfr is C-8 methylation, analysis of a *cfr*$^+$ Δ*rlmN* strain by mass spectrometry showed that Cfr has a secondary C-2 methylation activity, which is the primary activity of RlmN (25). Phylogenetic analysis of the RlmN/Cfr family of proteins suggests that the RlmN subfamily is the ancestral subfamily and that Cfr likely arose through duplication and horizontal gene transfer (37). Both RlmN and Cfr are radical *S*-adenosylmethionine (SAM) enzymes, a superfamily that catalyzes a diverse set of reactions that involve cleavage of unreactive C-H bonds by a 5′-deoxyadenosyl radical generated by reductive cleavage of SAM (82, 92). A recent crystal structure of the *E. coli* RlmN enzyme (4) and studies on the reaction mechanisms of RlmN and Cfr show that the methyl group is not transferred directly from SAM to the RNA but rather through a two-step sequence involving intermediate methylation of a conserved cysteine residue in the C-terminal domain (27).

The *cfr* gene was originally discovered on multiresistance plasmids isolated during surveillance studies of florfenicol resistance in *Staphylococcus* isolates of animal origin (38, 76). The first *cfr*-positive clinical strain of methicillin-resistant *S. aureus* was isolated in 2005, and it had *cfr* on the chromosome together with the *ermB* gene on a transposable genetic element (89). This strain is notable because these two rRNA methyltransferase genes are located on the same operon and their coexpression confers resistance to all clinically relevant antibiotics that target the large ribosomal subunit (81). A number of staphylococcal clinical isolates containing *cfr* in different genetic contexts and parts of the world have subsequently been reported (5, 6, 16, 17, 65, 67, 72, 80). In some instances, a connection between the resistant isolates and prior linezolid treatment (6, 64) or extensive use of linezolid (72) can be documented. In addition to staphylococci, the *cfr* gene has also been identified in two *Bacillus* strains isolated from swine feces (13, 102). Moreover, a recent report detects *cfr* in an isolate of *Proteus vulgaris*, a naturally occurring Gram-negative bacterium of pigs. The gene is found in a region with homology to a staphylococcal plasmid that is flanked by two IS*26* elements and inserted into the chromosomal *fimD* gene (93). The presence of *cfr* on mobile genetic elements such as plasmids and transposons in different geographical locations strongly suggests that it can be disseminated within the microbial community and spread among pathogenic bacteria.

DOES LINEZOLID RESISTANCE COME WITH A COST AND HOW IS IT RELATED TO CROSS-RESISTANCE?

The maintenance and spread of resistance genes are directly related to their fitness cost. Expression of Cfr in a laboratory strain has only a small effect on growth rate. Competition experiments involving wild-type and inactivated Cfr indicate that the small fitness cost is due not to the C-8 methylation but rather to expression of the protein (45). Similar experiments with strains coexpressing the Erm methyltransferase showed that dimethylation of 23S rRNA position A2058 increases the fitness cost of Cfr-mediated methylation of A2503 (45). The low fitness cost of *cfr* is troubling, as it suggests that cells can maintain the gene even in the absence of antibiotic selection. The available data on the fitness cost of RlmN and the effect of RlmN-mediated methylation on linezolid susceptibility are confusing and contradictory. It is concluded from one study that an RlmN-deletion strain is outcompeted by wild-type cells in the absence of antibiotic selection and that RlmN methylation leads to a 2-fold decrease in linezolid susceptibility in *S. aureus* (90). In a follow-up study, the authors concede that the earlier MIC data are inconclusive (44). Subsequent competition experiments show that cells with an inactivated *rlmN* gene outcompeted wild-type cells under linezolid selection (44). This result is corroborated by a study of clinical *S. aureus* strains (23) and has implications for resistance development in patients undergoing prolonged therapy with linezolid.

The fitness cost of ribosomal mutations varies enormously from one position to another and is also dependent on the specific organism. Some bacteria accept mutations at positions in 23S rRNA that are lethal in others. As mentioned above, a resistance mutation may be accompanied by other mutations that compensate for deleterious effects or act synergistically to enhance resistance (3). A decrease in growth rates for 23S rRNA mutations at the PTC is expected because many of the nucleotides are phylogenetically conserved and are considered functionally important. Among the engineered single mutations in *M. smegmatis* that have the most significant effects on linezolid resistance, the mutations lead to either moderate (A2503G/U and G2447U) or large (U2504G and G2576U) decreases in growth rate, where the G2576U mutation has the largest effect and results in 3-fold-slower growth (47, 56, 57). This is consistent with the fact that although both the G2447U and G2576U mutations lead to 32-fold increases in linezolid MIC values, only the G2447U mutation was isolated by selection in the presence of linezolid (56, 73). Engineered double mutations in *M. smegmatis* (G2032A-C2499A, G2032A-U2504G, C2055A-U2504G, and C2055A-A2572U) led to significantly slower growth compared to the corresponding single mutations, suggesting that multiple changes together have detrimental effects in this functionally important region of the ribosome (56). The G2576U mutation has been studied extensively in *S. aureus*, where a progressive decrease in growth rate is observed with each additional copy of the mutation (2). However, the abil-

ity of the mutation to persist in one copy in the absence of antibiotic selection and the rapid reemergence of multiple mutated copies upon reexposure to linezolid suggest that a single copy has a minimal fitness cost (91).

Cross-resistance between PTC antibiotics resulting from 23S rRNA mutations is not uncommon, but there is no straightforward relationship between overlapping binding sites and cross-resistance. Although the different sets of specific mutations, bacteria, and antibiotics examined in the literature preclude a detailed analysis, some patterns have emerged. There is a correlation between linezolid and chloramphenicol resistance for the single G2447U, A2503G, U2504G, G2505A, and G2576U mutations in *M. smegmatis* (56, 57). However, this correlation does not hold for the G2032A-U2504G and C2055A-U2504G double mutations, which both include the U2504G mutation (56). In the same studies, no relationship between linezolid, clindamycin, and valnemulin resistance could be observed (56, 57). Cross-resistance between linezolid and tiamulin has been documented for the G2447U and U2500A mutations in *E. coli* and the G2576U mutation in *E. coli* and *S. aureus* (66). The complexity of cross-resistance patterns between PTC antibiotics is likely due to the unique set of interactions that each bound antibiotic makes with the PTC cavity.

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